Supporting Information for

Designer Reagents for Mass Spectrometry-Based Proteomics: Clickable Cross-Linkers for Elucidation of Protein Structures and Interactions

*Chang Ho Sohn[1, 7], Heather D. Agnew [1, 7, 8], J. Eugene Lee[2], Michael J. Sweredoski[3], Robert L.J. Graham[3], Geoffrey T. Smith[3], Sonja Hess[3], Gregg Czerwieniec[4], Joseph A. Loo[5, 6], James R. Heath[1], Raymond J. Deshaies[2], and J. L. Beauchamp[1, *]*

[1]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

[2]Division of Biology, California Institute of Technology, Pasadena, CA 91125

[3]The Proteome Exploration Laboratory, Beckman Institute, California Institute of Technology, Pasadena, CA 91125

[4]Molecular Instrumentation Center, University of California, Los Angeles (UCLA), Los Angeles, CA 90095

[5]Department of Chemistry and Biochemistry, University of California, Los Angeles (UCLA), Los Angeles, CA 90095

[6]Department of Biological Chemistry, David Geffen School of Medicine, University of California, Los Angeles (UCLA), Los Angeles, CA 90095

[7] These authors contribute equally to this work.

[8]Current address: Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles (UCLA), Los Angeles, CA 90095 and

Integrated Diagnostics, 6162 Bristol Parkway, Culver City, CA 90230.

[*]To whom correspondence should be addressed at jlbchamp@caltech.edu.

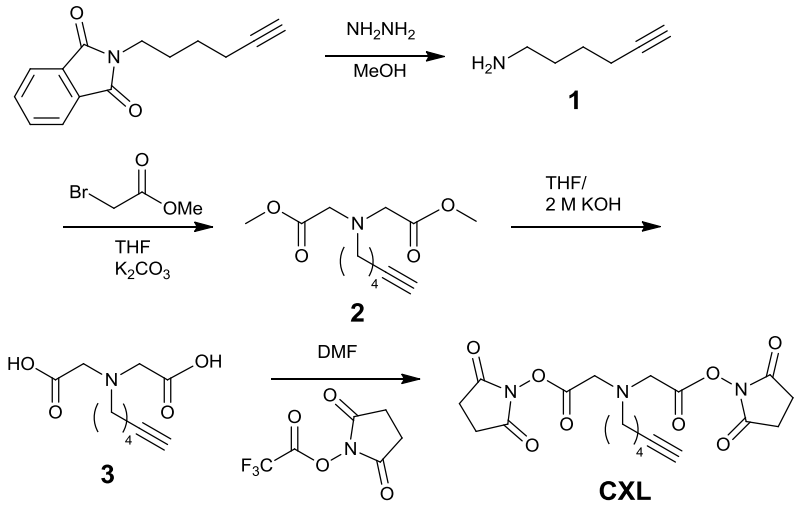
Table of Contents

1. Materials

N-(5-hexynyl)phthalimide, *N*-hydroxysuccinimide, methyl bromoacetate, trifluoroacetic anhydride, tetraethyl ammo nium bicarbonate, tris(2-carboxyethyl)phosphine, and bovine ubiquitin were obtained from Sigma-Aldrich Co. (St. Louis, MO). The hydrophilic ligand, tris[(hydroxyethyl-triazolyl)methyl]amine (TBTA-OH) was a kind gift from the Sharpless and Fokin groups at the Scripps Research Institute. The model peptides, Ac-AAKAAAAAKAR and Ac-AAAAKAAAAAR (98% purity), were obtained from Biomer-Tech (Pleasanton, CA). Yeast cell lysate was a generous gift from the Coon group at the University of Wisconsin, Madison. Monomeric avidin resin kit was purchased from Pierce (Rockford, IL). Biotin-$(PEG)_3$-azide was acquired from Berry & Associates, Inc. (Dexter, MI). Sequencing-grade trypsin was obtained from Promega (Madison, WI). OMIX-C18 desalting tips (100 μL scale) were purchased from Varian, Inc. (Palo Alto, CA). Microcon YM-3K spin filter units, nitrocellulose membrane, and C18-ZipTip (10 μL scale) were purchased from Millipore (Billerica, MA). Microspin SCX columns were acquired from the Nest Group, Inc. (Southborough, MA). High-capacity neutravidin agarose resin, n-dodecyl-*β*-D-maltoside, and supersignal west pico chemiluminescent substrate were from Thermo Scientific (Rockford, IL). Lysyl endopeptidase (LysC) was from Wako Chemicals USA (Richmond, VA). Cell culture reagents, Flip-In T-REx 293 cells, plasmids, and monoclonal antibodies for Cul1 (322400), Skp2 (323300), and Skp1 (323800) were from Invitrogen (Carlsbad, CA), and that of Cand1 (sc-10672) was from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid DNA containing the human Cul1 sequence was purchased from Open Biosystems (Huntsville, AL). MLN4924 was a generous gift from Millennium: The Takeda Oncology Company (Cambridge, MA). All other general chemicals for buffers were purchased from Fisher Scientific (Hampton, NH), EMD (Gibbstown, NJ), VWR International (West Chester, PA), and Sigma-Aldrich (St. Louis, MO). All chemicals mentioned above were used as received without further purification.

2. Synthesis of the Clickable Cross-Linker (CXL)

Overall synthetic steps are summarized in Scheme S1.



Scheme S1

6-Amino-hex-1-yne (1). The amino alkyne was prepared from 2-(hex-5-ynyl)isoindoline-1,3-dione and hydrazine as described in the literature without modification.[1] The crude product was purified by flash chromatography (silica gel, dimethylene chloride:methanol = 3:1 ~ 1:1) to yield 6-amino-hexyne as a pale greenish yellow oil. Yield: 30%. ^{1}H NMR spectra is reproduced with the previous report.[2] ESI-MS $[M+H]^+$ *m/z* 98.1.

Dimethyl 2,2'-(hex-5-ynylazanediyl)diacetate (2). The 6-amino-hex-1-yne (0.3 g) was added to the stirring solution of 20 mL THF, 2 eq K_2CO_3, and 2.4 eq methyl bromoacetate. The mixture was further stirred at room temperature for 3 h under a stream of dry N_2. The reaction was monitored by thin layer chromatography (TLC) using n-hexane:ethyl acetate (= 2:1) as the mobile phase, and the mixture was filtered after completion of the reaction. The filtrate was concentrated and purified by flash chromatography (silica gel, n-hexane:ethyl acetate = 1:1). The final product, dimethyl 2,2'-(hex-5-ynylazanediyl)diacetate, was concentrated by rotary evaporation and acquired as a transparent oil. Yield: 59%. ESI-MS $[M+H]^+$ *m/z* 242.1, ^{1}H NMR ($CDCl_3$) δ 3.71 (s, 6H), 3.56 (s, 4H), 2.73 (t, 2H), 2.22 (m, 2H), 1.94 (t, 1H), 1.57 (m, 4H).

2,2'-(Hex-5-ynylazanediyl)diacetic acid (3). To the obtained ~1.4 g dimethyl 2,2'-(hex-5-ynylazanediyl)diacetate was added 20 mL THF and 20 mL of 2 M KOH. The mixture was stirred overnight and monitored by TLC. The organic layer was separated, and the aqueous layer was quenched by addition of 20 mL of 2 M HCl. The solvent (H_2O) was completely removed by rotary evaporation, and the resulting solid was dissolved in acetonitrile (ACN). The insoluble KCl salt was filtered, and the filtrate was concentrated by rotary evaporation. The final product of free acid was obtained as a greasy transparent oil. Yield: quantitative. ESI-MS: $[M+H]^+$ *m/z* 214.1. To obtain the hydrochloride salt, an additional 10 mL of 2 M HCl was added before removal of the solvent. Dimethyl formamide (DMF, 3 × 30 mL) was added to the resulting solid and filtered. The hydrochloride salt was obtained as a white solid after concentration under reduced pressure. Yield: quantitative.

NHS-activated 2,2'-(hex-5-ynylazanediyl)diacetic acid (CXL). *N*-hydroxysuccinimide trifluoroacetate was prepared by stirring *N*-hydroxysuccinimide (NHS) and 4 eq trifluoroacetic anhydride for 5 h. The mixture was concentrated under reduced pressure and further dried under high vacuum overnight. The product was obtained as a white, highly hygroscopic solid and stored in an anhydrous desiccator before use. The obtained ~1.1 g 2,2'-(hex-5-ynylazanediyl)diacetic acid was activated by 2.4 eq *N*-hydroxysuccinimide trifluoroacetate in 10 mL anhydrous DMF under a stream of dry N_2. The mixture was stirred overnight and monitored by TLC using n-hexane:ethyl acetate (= 2:1) as the mobile phase. After completion of the reaction, the mixture was concentrated to ~500 µL by rotary evaporation and subjected to flash chromatography using n-hexane:ethyl acetate (= 2:1) as the mobile phase. The final product, NHS-activated 2,2'-(hex-5-ynylazanediyl)diacetic acid (CXL) was concentrated by rotary evaporation, and obtained as a pale yellow oil. Several 200 µL aliquots of 50 mM stock solution dissolved in anhydrous dimethyl sulfoxide (DMSO) were prepared and stored at –80 °C. The sealed stock aliquots were opened immediately before use, and NHS activation was verified by ESI-MS in 100% ACN. ESI-MS $[M+H]^+$ *m/z* 408.1, $[M+Na]^+$ *m/z* 420.0. (Note: the reaction yield can be improved by adding a stoichiometric equivalent of triethylamine for NHS activation.)

3. Cross-Linking of a Model Peptide

A 50 μg portion of the model peptide, Ac-AAKAAAAAKAR or Ac-AAAAKAAAAAR (98% purity) was dissolved in 50 μL of HPLC-grade H_2O. A mixture of 5 μL CXL stock solution (10 μg/μL in DMSO), 5 μL Ac-AAKAAAAAKAR or Ac-AAAAKAAAAAR stock solution (10 μg/μL), and 15 μL ACN was prepared. The mixture was allowed to react at room temperature for 1 h. The reaction was terminated by adding 5 μL formic acid (FA). The solvent was completely removed *in vacuo*, and the residue was reconstituted in 100 μL of 0.1% FA (aq) with additional 2 μL FA to further acidify. The resulting solution was desalted using an OMIX-C18 tip (100 μL capacity) following the standard procedure. The cross-linked peptide (~50 μg) was eluted in 100 μL solution of 0.1% FA, 50% ACN and 50% H_2O and 5 μL of the eluted cross-linked peptide solution was diluted to 5 μM and analyzed by a LCQ ion trap mass spectrometer. The remaining cross-linked peptide solution was dried for click reaction.

CuAAC with the biotin-(triethyleneglycol)-azide (biotin-$(PEG)_3$-azide) was performed as follows: Ten micrograms of the CXL cross-linked model peptide were dissolved in 100 mM tetraethyl ammonium bicarbonate (TEAB) (pH = 8.5), containing 250 μM hydrophilic ligand tris[(hydroxyethyl-triazolyl)methyl]amine (TBTA-OH), 2.5 mM $CuSO_4 \cdot 5H_2O$, 5 mM tris(2-carboxyethyl)phosphine (TCEP) hydrochloride and 1 mM biotin-$(PEG)_3$-azide in a total volume of 100 μL 1% (v/v) DMSO final. The mixture was reacted for 2 h with gentle mixing at 37 °C and quenched by 5% FA (aq). The solvent was removed *in vacuo* and the residue was desalted using OMIX-C18 tip as described above. The eluent was diluted to 10 μM with 0.1% FA, 50% ACN and 50% H_2O and directly infused to the LCQ ion trap mass spectrometer for analysis.

4. Cross-Linking of Ubiquitin

The general reaction summary of cross-linking of ubiquitin (PDB ID: 1UBQ) is shown in Scheme 1. Twenty micrograms of ubiquitin were dissolved in 200 µL of 1X PBS (pH = 7.4) and 1.2 µL of 50 mM CXL stock solution in DMSO was added and reacted for 30 min at room temperature. The reaction was quenched by 50 µL of 100 mM Tris-HCl buffer (pH = 8.5) and incubated for 15 min. The cross-linked ubiquitin was concentrated to ~30 µL and the buffer was exchanged to 100 mM ammonium bicarbonate (pH = 8.5) using Microcon YM-3K spin filter units. The trypsin digest reaction volume was adjusted by adding 185.5 µL of 100 mM ammonium bicarbonate buffer containing 2 M urea and 2.5 µL of 100 mM $CaCl_2$. Two microliters of 0.5 µg/µL trypsin in 5 mM acetic acid (proteins:trypsin = 20:1 w/w) was added and incubated for 15-18 h at 37 °C. The reaction was terminated by addition of 5% FA (aq). The resulting tryptic digest was desalted using OMIX-C18 tip and a 1 µg portion was injected into a nanoLC-LTQ-FTICR mass spectrometer for analysis.

Forty micrograms of the cross-linked tryptic digest of ubiquitin were subjected to click reaction by combining resulting peptides from two identical cross-linking experiments. The desalted tryptic digest was dissolved in 100 mM TEAB, containing 250 µM hydrophilic ligand TBTA-OH, 2.5 mM $CuSO_4 \cdot 5H_2O$, 5 mM TCEP hydrochloride and 1 mM biotin-$(PEG)_3$-azide in a total volume of 100 µL 1% (v/v) DMSO final. An additional sample was prepared by mixing 50 µg cross-linked digest of ubiquitin with 50 µg yeast cell lysate and subjected to identical click reaction conditions to demonstrate enrichment from a complex sample. Mixtures were reacted at 37 °C for 12 h with gentle shaking. Reactions were quenched by addition of 5% FA (aq).

Microspin SCX columns (200 µL scale, with 50 µL of the bed volume for SCX material) were used for removal of excessive TBTA-OH and biotin-$(PEG)_3$-azide. A 10 µg portion of the peptides from the click reaction (25 µL) was dried to completeness *in vacuo* and the residue was reconstituted with 0.5% FA, 5% ACN (aq). Microspin SCX columns were prepared by applying 4 bed volumes (200 µL) of methanol then H_2O, respectively. Activation of the SCX material was performed by treatment with 200

μL of 500 mM ammonium acetate for 1 h at room temperature. After activation, the spin columns were washed with H_2O and equilibrated with 0.5% FA, 5% ACN (aq). The peptide sample solution was applied to the spin column and flushed twice to bind completely. The spin column was washed with 400 μL of 0.5% FA, 5% ACN (aq), which corresponds to at least 8 bed volumes of the SCX material. The peptides were fractionated with 400 μL of 50, 250, and 500 mM ammonium acetate in 0.5% FA, 25% ACN (aq), and additional 500 mM ammonium acetate solution was used for complete elution of highly charged cross-linked peptides. Each fraction was desalted using C18-ZipTip following manufacturer instructions, and eluents were dried *in vacuo*. The residues were reconstituted with 5 μL of 0.2% FA (aq) and injected to a nanoLC-LTQ-FTICR mass spectrometer for analysis.

Monomeric avidin-biotin affinity chromatography was performed using the batch style procedure according to the manufacturer manual with minor modifications. Peptide samples after click reaction were eluted without SCX fractionation using either 500 mM ammonium acetate in 0.5% FA, 25% ACN (aq) or 50 mM ammonium acetate in 0.1% TFA, 25% ACN (aq). The SCX eluents were dried *in vacuo* and reconstituted with 1X PBS to the same concentration range used in the cross-linking reaction. By batch incubation of the mixture of the monomeric avidin resin and peptide samples at room temperature or 4 °C for 12 h under gentle mixing, the biotin-PEG_3-azide conjugated peptides were bound to monomeric avidin. Unmodified peptides were removed by flushing the resin with 4 bed volumes of PBS, 100 mM Tris-buffer (pH = 7.4), 100 mM ammonium bicarbonate (pH 7.4) and water, respectively. The final products of interest were eluted by 0.4% TFA, 50% ACN (aq). An aliquot of the eluent was analyzed by a nanoLC-LTQ-FTICR mass spectrometer.

5. *In vivo* Cross-linking of Cul1

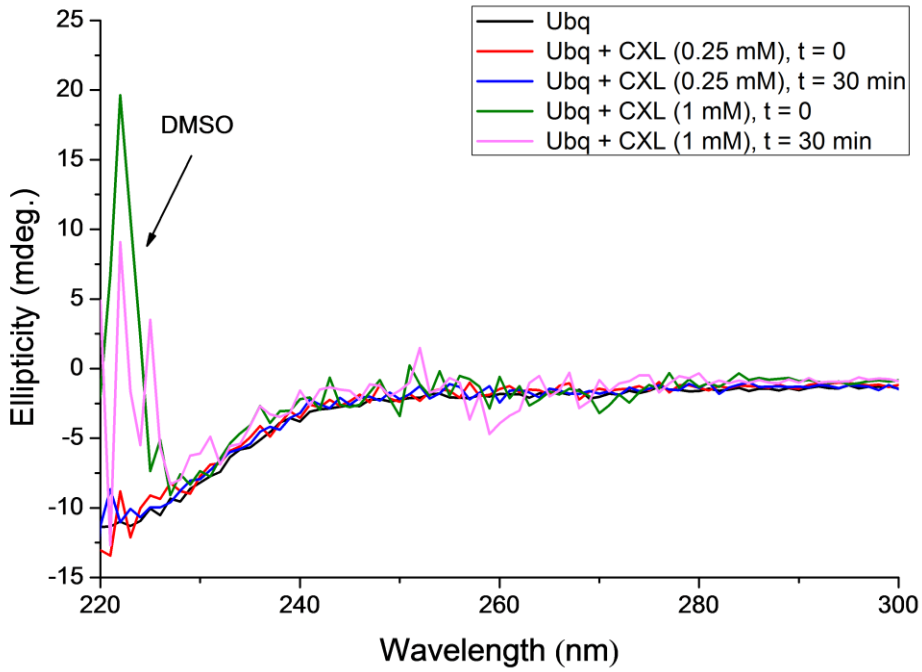
The *in vivo* cross-linking of HEK 293 cells by CXL, followed by Western blot analysis were carried out as described previously with minor modifications.[3] Briefly, to facilitate the purification of Cul1, a HEK 293-derived stable cell line capable of expressing tagged Cul1 upon tetracycline treatment was constructed using the T-REx^{TM} (Tetracycline-regulated Expression) system (Invitrogen, Carlsbad, CA). The tandem tag we adopted consists of a hexa-histidine sequence and a biotinylation signal sequence.[4] Biotinylation is catalyzed by endogenous biotin ligases, which are present in all eukaryotic cells.[5] A specific lysine residue in the biotinylation signal sequence functions as an acceptor site for biotin *in vivo*.[6]

Tagged Cul1 was induced with 1.0 μg/mL tetracycline for 4 h in experiments for *in vivo* cross-linking. Twenty four hours after induction, cells were washed by 1X PBS and subject to *in vivo* cross-linking by treatment with 0, 0.2, 0.5, and 1.0 mM of CXL, and 0.2 and 0.5 mM of disuccinimidyl suberate (DSS), respectively and incubated for 1 h at 37 °C. After completion of the cross-linking, unreacted cross-linkers were quenched by 12.5 mM glycine buffer in 1X PBS (pH = 7.4) for 10 min at room temperature. Cross-linked cells were harvested and lysed for 30 min at 4 °C with the lysis buffer (0.050 M HEPES, pH 7.5, 0.0050 M Mg(OAc)2, 0.070 M KOAc, 10% glycerol, and 0.4% IGEPAL CA630). The lysate was centrifuged at 16,600 g at 4 °C for 20 min and a portion of the supernatant was used for Western blot analysis against Cul1. The remaining lysates were immuno-precipitated with Neutravidin agarose resin (Thermo Scientific) for 1 h at room temperature followed by three washing steps with lysis buffer. Recovered Cul1^{THBH} and cross-linked proteins were eluted from the resin by boiling in 2× Laemmli sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membrane (Millipore). Proteins were detected with antibodies directed against Cul1 (322400, Invitrogen), Cand1 (sc-10672, Santa Cruz Biotechnology), Skp2 (323300, Invitrogen), or Skp1 (323800, Invitrogen) using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

6. Circular Dichroism Spectrometry

The CXL cross-linked ubiquitin samples were analyzed by an Aviv Model 62A DS Circular Dichroism (CD) Spectrometer (Lakewood, NJ) at room temperature. The critical instrumental parameters included: acquisition range = 200 to 300 nm, step size = 1.00 nm, averaging time = 5 s, bandwidth = 1 nm, and path length = 1 mm. One scan was recorded for each spectrum. For CXL cross-linked samples, 1 to 4 µL of 50 mM CXL stock solution in DMSO was added to 196 to 199 µL of 0.1 mg/mL ubiquitin in 1X PBS pH 7.4 to result in a final concentration of 0.25 to 1 mM CXL in 200 µL. CD spectra were collected upon addition of CXL (t = 0) and after 30 min of cross-linking reaction (t = 0.5 h).

Figure S1. CD spectra of CXL cross-linked ubiquitin.



CD provides a very useful and fast analytical technique for analysis of secondary structure, folding, and binding properties of peptides and proteins.[7] The CD spectra of native ubiquitin were previously recorded, yielding the secondary structure analysis of 6% α-helix, 10% β-sheet and 84% random structures, which can be observed as increasingly negative ellipticity over the range of 225 to 240 nm.[8] Figure S1 depicts the CD spectra of ubiquitin cross-linked by various concentrations and reaction times

of CXL. No significant changes are observed among ubiquitin samples that are native (black), cross-linked at 0.25 mM (red, t = 0; blue, t = 30 min) or 1 mM CXL (green, t = 0; pink, t = 30 min). Abundant peaks from 220 to 230 nm result from the increased DMSO proportion in 1 mM CXL cross-linked ubiquitin samples (from 1% to 4%). The CD signal fluctuation in 1 mM CXL experiments is mainly caused by light scattering due to the increased concentrations of small molecules such as the CXL cross-linker and DMSO.

7. Mass Spectrometry

The CXL cross-linked Ac-AAKAAAAAKAR and Ac-AAAAKAAAAAR model peptide were analyzed by a LCQ-deca XP ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The sample was directly infused by the standard electrospray ionization source with a constant flow at 3 μL/min. The critical instrumental parameters were set up as follows: the spraying voltage at 3.5 kV, capillary voltage at 39 V, capillary temperature at 275 °C, and tube lens voltage at −60 V. Fifty scans were recorded for each spectrum.

For collision induced dissociation, the cross-linked peptides from ubiquitin were analyzed by a nanoflow HPLC (Waters Co.) coupled on-line via a home-built nanoelectrospray ion source to a LTQ-FTICR mass spectrometer (Thermo Fisher Scientific). Samples in 5 μL of 0.2% FA (aq) were loaded onto a C18-reversed phase column (15 cm long, 100 μm inner diameter, packed in-house with Magic C18-AQ 5 μm resin from Michrom Bioresources, in solvent A (2% ACN, 0.2% FA) with a flow rate of 250 nL/min for 24 min and eluted with a linear gradient from 0% to 36% solvent B (98% ACN, 0.2% FA) over 110 min, followed by 10 min at 100% solvent B, at a flow rate of 250 nL/min. The column was re-equilibrated with solvent A. Mass spectra were acquired in the positive ion mode applying data-dependent acquisition with automatic switching between survey scan and tandem mass spectrum acquisition. Samples were analyzed with a "top 10" method; acquiring one FTICR survey scan in the mass range of *m/z* 400–1600 followed by MS/MS of the ten most intense ions in the LTQ. The target ion value in the LTQ-FTICR was 500,000 for survey scan at a resolution of 50,000 at *m/z* 400. Fragmentation in the LTQ was performed by CID with a target value of 5,000 ions. Selected sequenced ions were dynamically excluded for 30 s. Critical mass spectrometric parameters were: spray voltage, 2.4 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 200 °C; normalized collision energy (35%) for MS/MS.

For electron transfer dissociation, cross-linked peptide samples were loaded and washed on a 100 μm × 35 mm CVC MicroTech (Fontana, CA) C18 trap before chromatographic separation was performed

on a New Objective Halo C18 75 μm × 100 mm, 90 Å, 2.7 μm (Woburn, MA) column using mobile phases of 0.1% FA in water (solvent A) and ACN containing 0.1% FA (solvent B). Peptides were eluted from the column at a flow rate of 300 nL/min using an Eksigent nanoLC 2D pump (Dublin, CA) with a 110 min gradient (0-30% phase B over 90 min, 30-80% phase B over 20 min). The HPLC system was coupled to an LTQ-Orbitrap XL mass spectrometer and the source conditions were as follows: capillary temperature, 180 ºC; capillary voltage, 49 V; ESI spray voltage, 1.8 kV. The automatic gain control target was fixed at 5×10^5 ions for MS and 1×10^4 for MS/MS scans. The instrument was operated in the data-dependent acquisition (DDA) mode, with MS survey scan (*m/z* 300-2000) performed in the Orbitrap using a resolution set at 60,000. Parent ions with ions of charge state +1 were rejected for DDA MS^2 using charge-state screening. CID activation was performed on the 4 most abundant ions over 10,000 counts using normalized collision energies of 35, an activation Q of 0.250, and an activation time of 30 ms followed by detection using the linear ion trap. ETD activation was also performed on the 4 most abundant ions (over 50,000 counts) using a reaction time of 125 ms, a default charge state of +2, and charge state dependent ETD time with supplemental activation enabled. The ETD emission current used was 785 μA, and the electron energy was set at 70 eV. The CI gas pressure and source temperature were 9 psi and 160 ºC, respectively. Ions with masses within 10 ppm of previously fragmented ions were excluded from DDA for 30 s.

8. LC-MS Elution Profiles Analysis

Sample Clean-up Following Click Reaction

CuAAC is a widely used bioconjugation reaction. However, seamless integration of CuAAC into the downstream proteomics workflows can be challenging due to the persistence of residual chemical reagents such as copper ions, hydrophobic ligands, and coupling reagents (azide or alkyne).[9,10] Those impurities often adversely impact the ionization efficiency of target peptides. Effective sample clean-up procedure after CuAAC is therefore very important for successful sample analysis by MS.

For this purpose, we adopted SCX, followed by C18 desalting procedures for the removal of non-ionic species following CuAAC. The hydrophobic ligand reagent also has been replaced by its hydrophilic counterpart, tris[(hydroxyethyl-triazolyl)methyl]amine (TBTA-OH). Poor binding of TBTA-OH to the C18 matrix would result in its effective removal *via* C18 desalting column. Copper ions can be removed during the same desalting step.

Figures S2a-c and Figure 4 in the main text show LC-MS profiles of ubiquitin cross-linked peptide samples following CuAAC. TBTA-OH was not detected in any LC-MS profile, confirming its successful removal. Excess biotin-$(PEG)_3$-azide molecules were mostly removed by SCX clean-up due to their poor interaction with the SCX matrix, but were not completely eliminated (Figures S2a-c). The protonated biotin-$(PEG)_3$-azide (*m/z* 445.2) was eluted along with peptides (retention time around 55 min in Figure S2a and 46 min in Figures S2b and S2c), but the mass-to-charge ratio did not overlap with those of other cross-linked peptides, ensuring no interference with the sequencing. Based on its ion signal, the residual amount of biotin-$(PEG)_3$-azide after SCX clean-up was tolerable as it did not significantly suppress peptide ionization (Figures S2b and S2c), especially for cross-linked peptides that were eluted mostly in the SCX fractions at 250 and 500 mM salt concentrations (Table S2). For complete removal of the residual azide affinity tags, cleavable biotin tags[11] can be employed along with streptavidin-conjugated magnetic resin, which is expected to suffer less nonspecific binding than

monomeric glycosylated avidin. This may allow us to enhance the elution purity *via* the chemical cleavage of affinity tags.

Peptide Fractionation by SCX

Highly-charged species tend to more strongly bind to SCX matrix. Therefore, SCX can be used for sample fractionation by discriminating the charge states of analytes. Primary sample fractionation by SCX was demonstrated for sensitive detection of cross-linked peptides from abundant linear peptides by Rinner et al.[12,13] For the present study, peptide fractionation of ubiquitin cross-linked peptides was performed by sequential increase of the salt concentration during the SCX elution step. Figures 4a-e in the main text show LC-MS total ion current (TIC) chromatograms of differentially eluted ubiquitin cross-linked peptide samples by applying salt gradients, 50 mM, 250 mM, 1st 500 mM, and 2nd 500 mM ammonium acetate, 0.5% FA, respectively (Figure 4b-e), or direct elution using 500 mM ammonium acetate, 0.5% FA with no fractionation (Figure 4a). As summarized in Tables S1 and S2, highly charged cross-linked peptides were eluted in the high concentration region. However, some of the cross-linked peptides were also co-eluted with other linear peptides at 250 mM salt concentration (Table S2, Figure 4c). Thus, marginal separation of cross-linked peptides was achieved by SCX fractionation. For further optimization of the separation, (an) additional elution step(s) using intermediate salt concentrations between 50 to 250 mM can be performed. Due to the low complexity of the ubiquitin cross-linked sample, SCX fractionation by itself is sufficient for separation and identification of cross-linked peptides from other linear peptides (Figures 4a-e, Tables S1 and S2). As pointed out by Rinner *et al.*,[12] however, the ultimate test for enrichment capability is found in the application of CXLs to higher complex systems such as *in vivo* cross-linking in eukaryotic cells.

Figure S2a. LC-MS elution profiles of ubiquitin cross-linked, clicked and SCX clean-up (single elution by 500 mM salt)

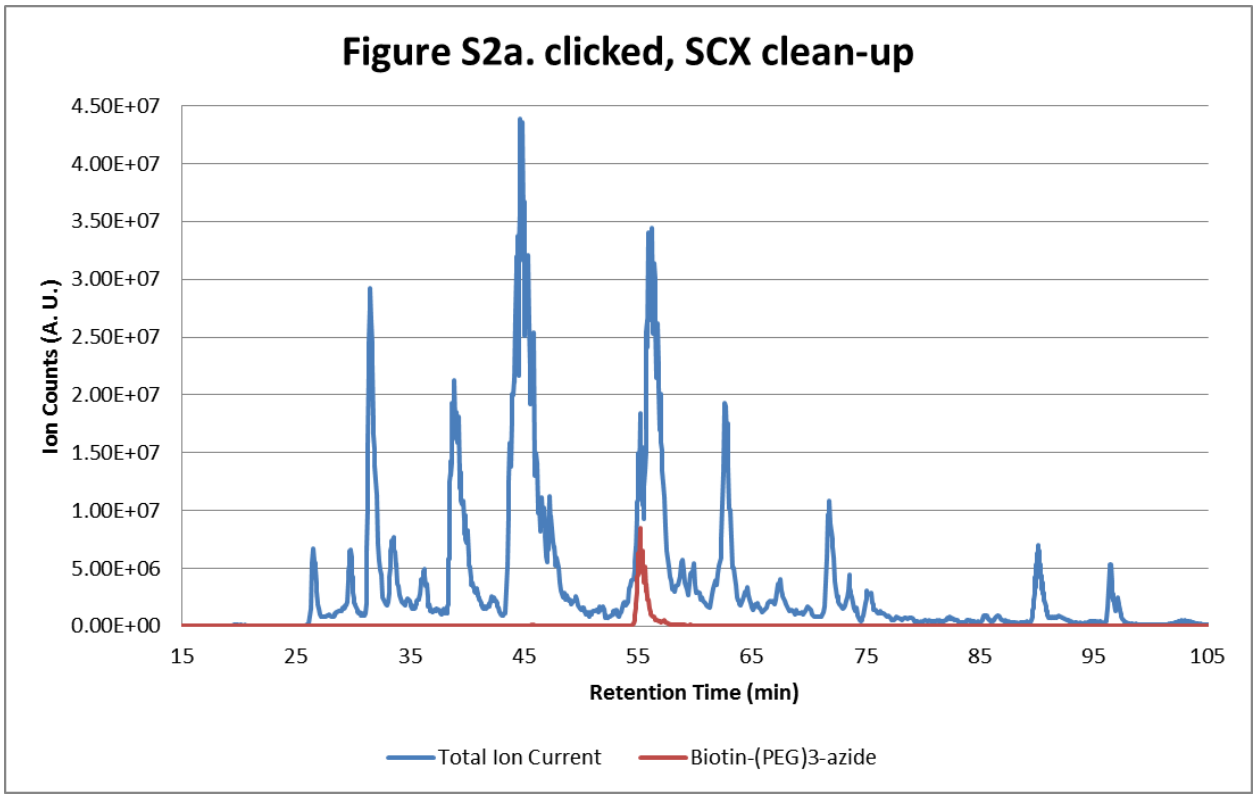


Figure S1b. LC-MS elution profiles of ubiquitin cross-linked, clicked and SCX 50 mM elution

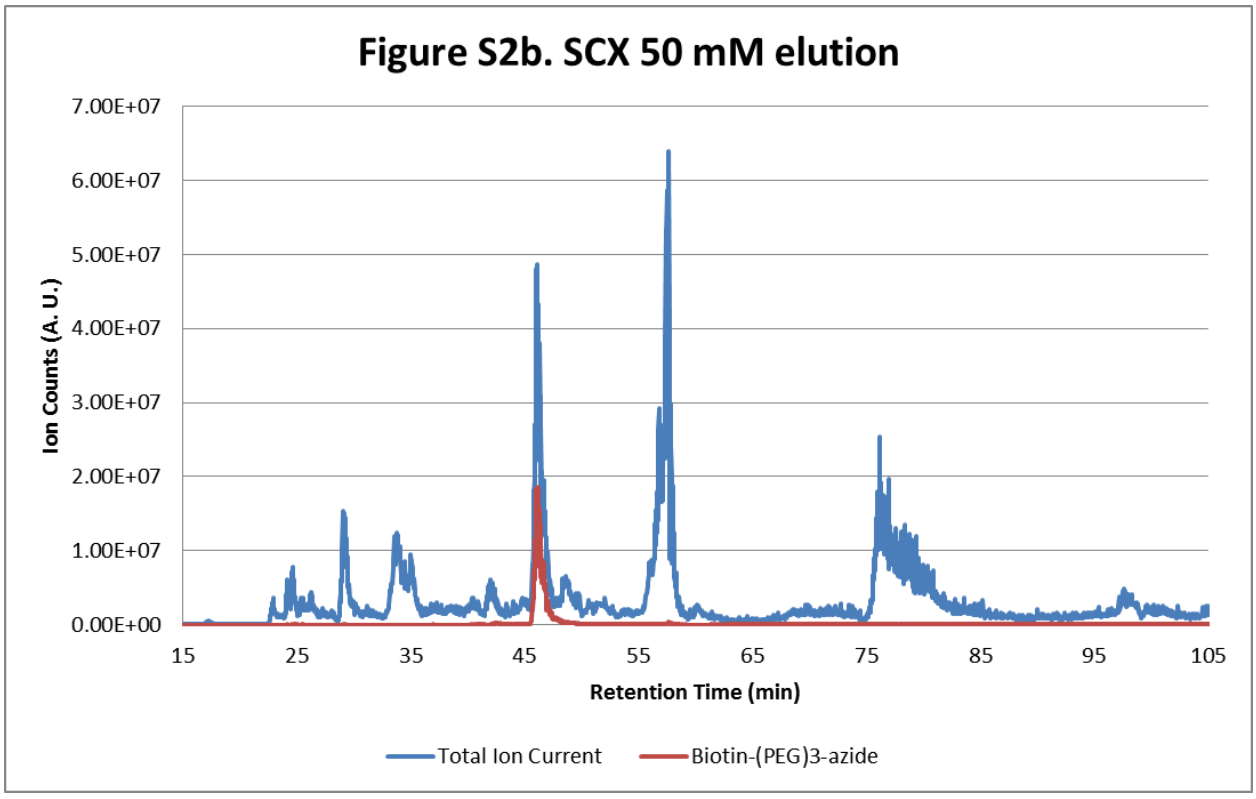
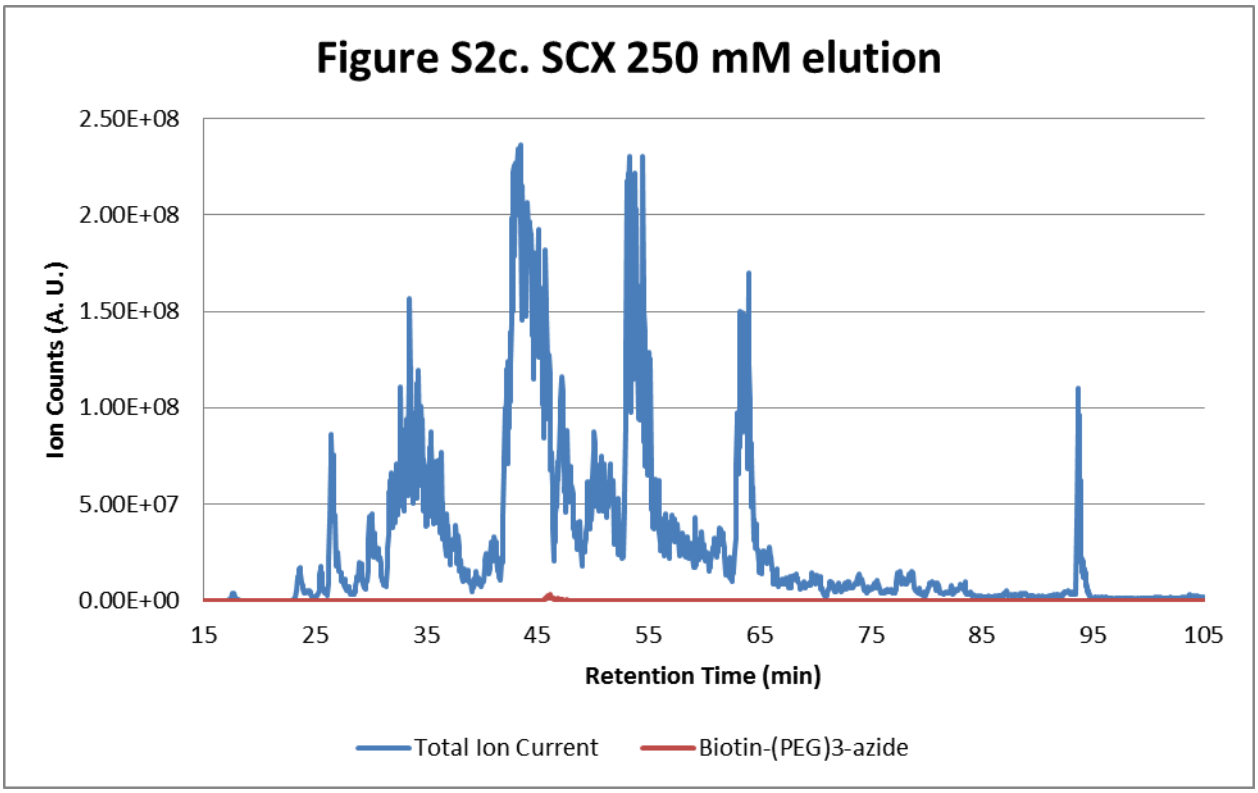


Figure S2c. LC-MS elution profiles of ubiquitin cross-linked, clicked and SCX 250 mM elution



9. xQuest Search

The raw files from the LTQ-FTICR mass spectrometer were converted to Mascot generic format (MGF) files using ReAdW4Mascot (version 20090305a, available from the National Institute of Standards and Technology at http://peptide.nist.gov/software/ReAdW4Mascot2_20090305a.zip), and all but the 150 most intense fragment ions were filtered out. The cross-linked peptide search was performed using xQuest (http://prottools.ethz.ch/orinner/public/htdocs/xquest)[12]. The database search parameters were as follows: 0.5 Da fragment ion mass tolerance; 0.3 Da common fragment ion mass tolerance; 10 ppm precursor ion mass tolerance; trypsin enzyme specificity (up to two missed cleavages); fixed carbamidomethyl (57.02146 Da) modification of cysteine; and variable modifications of methionine oxidation (15.99491 Da), cross-linked primary amines (177.07898 Da), mono-linked dead ends (195.08954 Da), cross-linked and biotin-$(PEG)_3$-azide clicked primary amines (621.295568 Da), mono-linked and biotin-$(PEG)_3$-azide clicked primary amines (639.305584 Da), and reporter ion (525.28537 Da). Both reporter ion-filtered and non-filtered MGF files by xQuest search were tested, but identical results were reported. Reduced MS/MS scans for xQuest search resulted in faster searching for the reporter ion-filtered MGF files.

10. X-ray Crystal Structure Analysis

Human ubiquitin (PDB ID: 1UBQ. Note: the sequences of human and bovine ubiquitin are identical.) structure was analyzed by UCSF Chimera (version 1.5.3rc).[14] For each pair of cross-linked lysine residue, the distances between the alpha carbons (C_α–C_α) and between nitrogens of ε-amines (NZ–NZ) were measured.

11. Tables of Cross-linked Peptides from xQuest Search

Modified lysines in mono-linked peptides indicate solvent accessible residues. Intrapeptide cross-linked peptides are generated by cross-linking of two lysine residues that are closely positioned, and which contain no unmodified lysine or arginine residues between them for tryptic cleavage. Interpeptide cross-linked peptides have one or more unmodified cleaved lysine or arginine residues between the cross-linked lysine residues, yielding two peptide chains covalently joined by the cross-linker. These interpeptide cross-linked peptides constrain the topology of surface exposed lysine residues, which is of particular interest for the construction of low resolution three-dimensional protein structures. Following tables are the summary of dead-end, intrapeptide and interpeptide cross-linked peptides observed from ubiquitin cross-linking experiments.

Table S1. Dead-end and Intrapeptide Cross-linked Peptides from Ubiquitin.

Sequence	Before Click	After Click & SCX Fractionation	Reporter Ion
^{1}MQIFV^{6}K^TLTG^{11}K	Yes, 2+, 3+	Yes, 2+, 3+, 250 mM	Yes
^{7}TLTG^{11}K^TITLEVEPSDTIENV^{27}K	Yes, 2+, 3+, 4+	Yes, 3+, 4+, 250 mM	Yes
^{12}TITLEVEPSDTIENV^{27}K^A^{29}K	Yes, 3+	Yes, 3+, 250 mM	Yes
^{28}A^{29}K^IQD^{33}K	Yes, 2+	No	N/A
^{28}A^{29}K^IQDKEGIPPDQQ^{42}R	Yes, 3+	No	N/A
^{28}AKIQD^{33}K^EGIPPDQQ^{42}R	Yes, 3+	Yes, 4+, 250 mM	Yes
^{30}IQD^{33}K^EGIPPDQQ^{42}R	Yes, 2+, 3+	Yes, 2+, 3+, 250 mM	Yes
^{43}LIFAG^{48}K^QLEDG^{54}R	Yes, 2+, 3+	Yes, 2+, 3+, 4+, 50, 250 mM	Yes
^{43}LIFAG^{48}K^QLEDGRTLSDYNIQ^{63}K	Yes, 2+, 3+, 4+	Yes, 3+, 4+, 250 mM	Yes
^{55}TLSDYNIQ^{63}K^ESTLHLVL^{72}R	Yes, 2+, 3+	Yes, 3+, 4+, 250 mM	Yes
^{1}MQIFV^{6}K^TLTG^{11}K^	Yes, 2+, 3+	Yes, 2+, 3+, 250 mM	Yes
^{28}A^{29}K^IQD^{33}K^EGIPPDQQ^{42}R	Yes, 2+, 3+	Yes, 3+, 250 mM	Yes

^: linked residues. Superscript numbers are the residue numbers of amino acids of ubiquitin.

Table S2. Interpeptide Cross-linked Peptides from Ubiquitin.

α chain	β chain	Before Click	After Click & SCX Fractionation	Reporter Ion	Avidin Enrichment	C_α distance (Å)	NZ distance (Å)
^{43}LIFAG^{48}K^QLEDG^{54}R	^{1}MQIFV^{6}K^TLTG^{11}K	Yes, 3+, 4+, 5+	No	N/A	No	15.1	17.8
^{1}MQIFV^{6}K^TLTG^{11}K	^{55}TLSDYNIQ^{63}K^ESTLHLVL^{72}R	Yes, 5+, 6+	No	N/A	No	15.0	20.3
^{30}IQD^{33}K^EGIPPDQQ^{42}R	^{7}TLTG^{11}K^TITLEVEPSDTIENV^{27}K	Yes, 4+, 5+	Yes, 4+, 5+, 250 mM, 1st 500 mM, 2nd 500 mM	Yes	Yes, 4+, 5+	12.9	7.2
^{43}LIFAG^{48}K^QLEDG^{54}R	^{43}LIFAG^{48}K^QLEDG^{54}R	Yes, 3+, 4+, 5+	Yes, 5+, 2nd 500 mM	Yes	Yes, 5+	N/A	N/A
^{43}LIFAG^{48}K^QLEDG^{54}R	^{55}TLSDYNIQ^{63}K^ESTLHLVL^{72}R	Yes, 6+	No	N/A	No	17.9	19.9
^{28}A^{29}K^IQD^{33}K	^{30}IQD^{33}K^EGIPPDQQ^{42}R	No	No	Yes	Yes, 5+	6.2	9.1

^: cross-linked residues. Superscript numbers are the residue numbers of amino acids of ubiquitin.

12. The Mechanism of Electron Transfer Dissociation of Cross-linked Peptides

In the previously reported cross-linker by Chowdhury et al. (Click-enabled Linker for Interacting Proteins, or CLIP),[15] the nitro group (NO_2) is inserted for water solubility, and neutral loss of NO_2 by CID can be used as a diagnostic peak. During ETD of cross-linked multiply charged peptide ions, due to the high hydrogen affinity of the nitro group, a nascent hydrogen atom produced by electron transfer can be immobilized, inhibiting the normal sequence of the fragmentation process.[16] As model systems of CLIP and its hydrogen adduct, nitromethane (CH_3NO_2), its hydrogen attached radical ($CH_3NO_2H\cdot$) and hydrogen atom were chosen. Initial geometry optimization and thermochemical calculations were performed by the B3LYP functional with the 6-311++G(d,p) basis set using Jaguar 7.5 (Schrödinger Inc., Portland, OR, USA). For odd electron species, open-shell configuration was employed. The refinement of single point energy was performed using (Q-Chem Inc., Pittsburg, PA, USA) at the CCSD(T)/6-311++G(d,p) level of theory. Enthalpies of model systems were calculated from electronic energy, zero-point energy and thermal contribution at 1 atm and 298.15K.

The resulting nitronic radical stabilizes the charge-reduced species and prevents further fragmentation to form c- and z-type ions, yielding abundant charge-reduced species. With CXLs, there are no occurrences of specific chemical bonds or residues that can generate unexpected neutral losses or stable charge-reduced species in ETD, thus allowing efficient electron based dissociation processes to dominate. The enthalpy change in hydrogen attachment to nitromethane was obtained from enthalpy of each species, yielding 164 kJ/mol, which is larger than those of amide carboxyls (21-41 kJ/mol).[17] The adiabatic electron affinity of niromethane was determined to be 0.172 ± 0.006 eV,[18] which is not high enough to trap an electron in electron capture/transfer dissociation (ECD/ETD) of peptide ions.[16] Therefore, an electron from ECD or ETD to the CLIP cross-linked peptide ions is initially captured in high-lying Rydberg states, and undergoes the relaxation process to low-lying Rydberg states of positive charges (the Cornell mechanism) or Columb stabilized π^* states of amide bonds (the Utah-Washington mechanism) *via* intermolecular electron transfer with internal conversion. Hydrogen transfer from a

hypervalent radical or proton transfer from any protonated sites yields aminoketyl intermediates whose bond dissociation energy (O–H) is much lower than the hydrogen affinity of nitromethane. Finally a labile hydrogen is scavenged by the nitromethane group, producing the relatively stable methyl nitronic radical in the charge-reduced cross-linked peptide ion.[16,19-21] This leads to the inhibition of normal backbone fragmentation processes observed in ECD and ETD, yielding the poor sequence coverage. The recent studies have shown that ECD of highly charged (>2+) nitrated peptides and proteins produces c- and z-type backbone fragments with diminished intensities.[22-25] This provides an explanation on the observation of some c and z ions in the ETD spectra of CLIP cross-linked peptides.

13. References

(1) Nilsson, B. L.; Overman, L. E.; Read de Alaniz, J.; Rohde, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 11297.

(2) Rozkiewicz, D. I.; Janczewski, D.; Verboom, W.; Ravoo, B. J.; Reinhoudt, D. N. *Angew. Chem.-Int. Edit.* **2006**, *45*, 5292.

(3) Lee, J. E.; Sweredoski, M. J.; Graham, R. L. J.; Kolawa, N. J.; Smith, G. T.; Hess, S.; Deshaies, R. J. *Mol. Cell. Proteomics* **2010**, *Electronic preprint. doi:10.1074/mcp.M110.006460*.

(4) Tagwerker, C.; Flick, K.; Cui, M.; Guerrero, C.; Dou, Y. M.; Auer, B.; Baldi, P.; Huang, L.; Kaiser, P. *Mol. Cell. Proteomics* **2006**, *5*, 737.

(5) Cronan, J. E. *J. Biol. Chem.* **1990**, *265*, 10327.

(6) Kulman, J. D.; Satake, M.; Harris, J. E. *Protein Expr. Purif.* **2007**, *52*, 320.

(7) Greenfield, N. J. *Nat. Protocols* **2007**, *1*, 2876.

(8) Jenson, J.; Goldstein, G.; Breslow, E. *Biochim. Biophys. Acta* **1980**, *624*, 378.

(9) Vellucci, D.; Kao, A.; Kaake, R. M.; Rychnovsky, S. D.; Huang, L. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 1432.

(10) Lallana, E.; Riguera, R.; Fernandez-Megia, E. *Angew. Chem.-Int. Edit.* **2011**, *50*, 8794.

(11) Szychowski, J.; Mahdavi, A.; Hodas, J. J. L.; Bagert, J. D.; Ngo, J. T.; Landgraf, P.; Dieterich, D. C.; Schuman, E. M.; Tirrell, D. A. *J. Am. Chem. Soc.* **2010**, *132*, 18351.

(12) Rinner, O.; Seebacher, J.; Walzthoeni, T.; Mueller, L.; Beck, M.; Schmidt, A.; Mueller, M.; Aebersold, R. *Nat. Methods* **2008**, *5*, 315.

(13) Lauber, M. A.; Reilly, J. P. *Anal. Chem.* **2010**, *82*, 7736.

(14) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. *J. Comput. Chem.* **2004**, *25*, 1605.

(15) Chowdhury, S. M.; Du, X.; Tolić, N.; Wu, S.; Moore, R. J.; Mayer, M. U.; Smith, R. D.; Adkins, J. N. *Anal. Chem.* **2009**, *81*, 5524.

(16) Sohn, C. H.; Chung, C. K.; Yin, S.; Ramachandran, P.; Loo, J. A.; Beauchamp, J. L. *J. Am. Chem. Soc.* **2009**, *131*, 5444.

(17) Turecek, F.; Syrstad, E. A. *J. Am. Chem. Soc.* **2003**, *125*, 3353.

(18) Adams, C. L.; Schneider, H.; Ervin, K. M.; Weber, J. M. *J. Chem. Phys.* **2009**, *130*, 074307.

(19) Belyayev, M. A.; Cournoyer, J. J.; Lin, C.; O'Connor, P. B. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1428.

(20) Jones, J. W.; Sasaki, T.; Goodlett, D. R.; Turecek, F. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 432.

(21) Tureček, F. *J. Mass Spectrom.* **2010**, *45*, 1280.

(22) Jones, A.; Mikhailov, V.; Iniesta, J.; Cooper, H. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 268.

(23) Jones, A. W.; Cooper, H. J. *Phys. Chem. Chem. Phys.* **2010**, *12*, 13394.

(24) Mikhailov, V. A.; Iniesta, J.; Cooper, H. J. *Anal. Chem.* **2010**, *82*, 7283.

(25) Cook, S.; Jackson, G. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 221.